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Initiation of Spermatophore Protrusion Behavior in the Male Cricket *Gryllus bimaculatus* DeGeer

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ABSTRACT—Spermatophore protrusion is the reproductive act of the male cricket ejecting the immature spermatophore to the external genitalia to solidify. Forty-five seconds prior to this act spermatophore materials are secreted into the ejaculatory duct. Initiation of spermatophore protrusion was facilitated by contact with the female: the interval between the end of copulation and spermatophore protrusion was considerably shorter in the male with the female (6 min) compared to the male with no female (>60 min) after copulation. It was also accelerated to a similar extent through increased sensitivity to the female when males were deprived of females for at least 12–24 hr prior to testing. On the other hand, the occurrence of spermatophore protrusion was delayed or prevented when the male was restrained in an abnormal posture. Spermatophore protrusion still occurred, though more slowly, in males with the connectives transected between the thorax and the abdomen.

INTRODUCTION

During copulation, the male insect gives the female a spermatophore, a proteinous capsule containing sperm. In the cricket *Gryllus bimaculatus*, the male starts to prepare a new spermatophore for the next mating about 6 min after copulation [1]: the white milky mass is ejected from the genital cavity into a space between the ventral lobes of the external genitalia. This act is called spermatophore protrusion. Once it occurs the male begins to sing a calling song at a fixed interval [2–5]. Thus, spermatophore protrusion is taken as the first step in the sequence of the male cycle.

Although the structure and formation of spermatophores of insects have been studied extensively [6–8], the initiation mechanism of spermatophore protrusion is not yet known. Behavioral study indicates that the female does not play a role in stimulating the initiation of spermatophore protrusion [3]. Our previous study showed that the interval (CPPT) between the end of copulation and spermatophore protrusion varied among males [5]. CPPT was extremely long in some males [5], and considerably prolonged by adverse stimulation of the genitalia [1, 4]. These suggest that the process of spermatophore protrusion may be affected by some external and internal factors which is different from the process of the calling song starting with a constant latency after spermatophore protrusion [2–5]. On the other hand, physiological study indicates that communication between the brain and the terminal abdominal ganglion was indispensable for spermatophore protrusion [9]. In particular, the pars intercerebralis of the brain seemed to be essential because its coagulation abolished this behavior [10]. There is, however, a conflicting result that elimination of the corpora allata or the corpus cardiacum, which is the terminal regions of the projection neurons in the pars intercerebralis [11], failed to abolish spermatophore protrusion [2].

Thus, we investigated external and internal factors that may affect the initiation of spermatophore protrusion and estimated nervous communication between the brain and the terminal abdominal ganglion.

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MATERIALS AND METHODS

Animals

Crickets *Gryllus bimaculatus* DeGeer reared at a 12L:12D photoperiod at 27±2°C were used. We previously reported CPPT varied considerably among individuals reared alone and gradually declined as males matured [5]. In the present study, we used males reared in a group because a large number of insects were used for various experiments. The daily change in CPPT (Fig. 1A) was checked in males (n=30) reared in a group with females (n=60) in a container (45×30×25 cm). CPPT was much longer and more variable, as compared with that in males reared individually isolated (Fig. 1B) and those (n=30) reared in a group with males (Fig. 1C) for the first 6 days after molt and then reached a constant level after day 8. Thus, crickets aged 8–10 days after molt were used. Males reared in the two latter conditions (B and C) were not used here except in only one experiment.

Behavior observation and data analysis

The male and female were paired in a 200 ml beaker. Behavioral events were checked by eye, but antennal and abdominal movements were recorded on video tape and later analyzed by slow speed play back [1]. After they copulated once, the time of reproductive events, i.e. the pairing of male and female, beginning of the calling song, spermatophore extrusion during copulation, the opening of the subgenital plate and spermatophore protrusion was recorded with a stop watch. In the present experiments, the end of copulation was

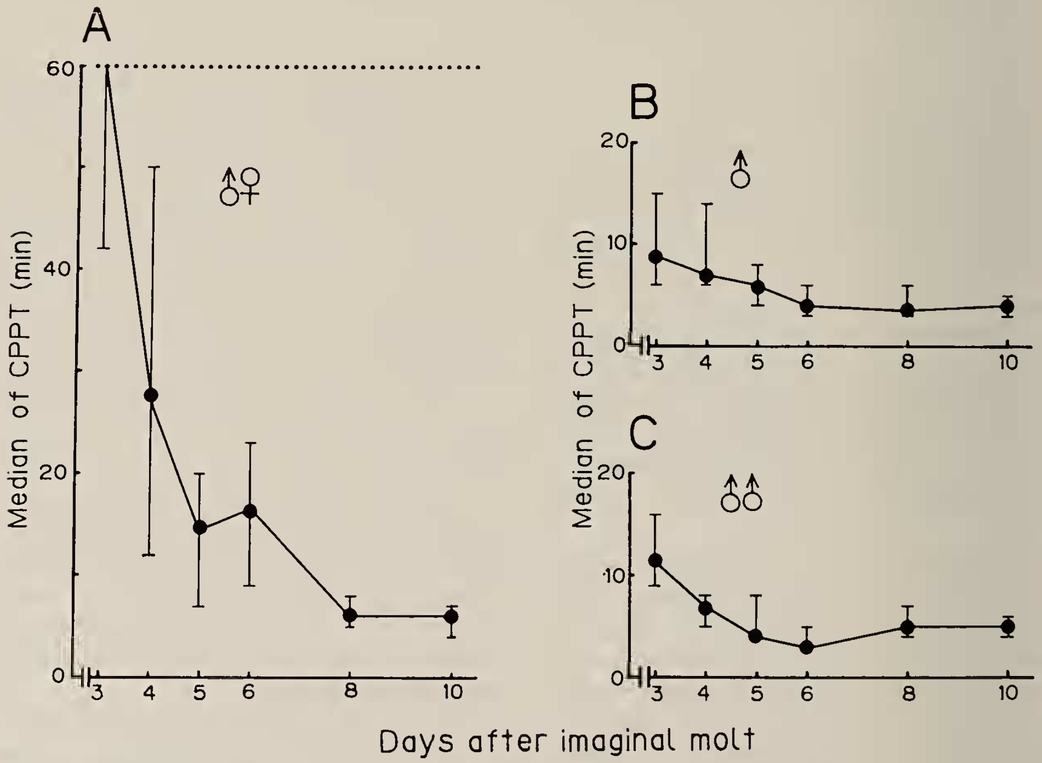

Fig. 1. Developmental changes in CPPT of spermatophore protrusion in males reared under 3 different conditions after molt. A: males reared in a group with females. B: males reared alone. C: males reared in a group with males. Each experiment started with 30 males but the number reduced to 29 in A and C, and 24 in B on day 10th owing to death. Each point represents the median value of CPPT and each bar, the 95% confidence interval (C.I.). The dotted line in A indicates the 1 hr observation time limit. Some of the same conventions are adopted in the subsequent figures.

defined as the moment of genitalia separation (GS in Fig. 2) which regularly occurred 10 sec after spermatophore extrusion [1], i.e., extrusion of the attachment plate of the spermatophore from the dorsal pouch [1]. Spermatophore protrusion was defined as the moment of ejection of an immature spermatophore from the genital cavity (SPPT in Fig. 2). Thus, CPPT is represented as the interval between GS and SPPT. CPPT was measured in minutes rounding off fractions to one decimal place. Data were expressed as medians with 95% confidence intervals (C.I.). Statistical significance on CPPT was set at $P<0.05$ by the Mann-Whitney U test unless otherwise stated.

Separation from the female

To examine the effect of the female on CPPT, two different procedures were used.

During testing: When copulation was accomplished, the female was removed from the beaker ("male" condition) and CPPT was timed. For comparison, the female was replaced with a male ("male and male" condition). Control was the "male and female" condition in which the male and female were simply left after copulation.

Prior to testing: All females were temporarily removed from the container before experiments and males experienced female-deprivation for 4, 8, 12, 24 or 72 hr prior to pairing. According to Fig. 1C, CPPT was expected to be shortened by these treatment. They were tested in the "male" condition. The reason for this will be described later.

Modifications of these procedures will be described in the places concerned.

Restraint of males

In order to examine whether spermatophore protrusion could occur in abnormal conditions, males were restrained in four different conditions immediately after copulation (Table 2). 1) Posture normal, legs contacted; the male was glued by the dorsal pronotum to a metal bar in a normal posture and placed on a movable styrol ball. 2) Posture normal, legs restrained; each tarsus of the 6 legs was fixed to the substrate with a piece of Scotch tape. 3) Posture reversed, legs contacted; the male was glued by the dorsal pronotum to a wooden mound with the ventral side up. A wooden plate was set over the male to contact with the legs on it. 4) Posture reversed, legs suspended; the same as in 3) except for no plate. All the males were given a periodic brushing of their antennae with female's antennae for 2–3 sec every 40 sec as female stimulation. If the male did not show spermatophore protrusion within a 1 hr observation period, it was checked again after 24 hr during which they were left without female stimulation.

Inspection of the spermatophore in the ejaculatory duct

The ejaculatory duct was examined for an immature spermatophore in males at 4 different phases of the male cycle (Table 1); after spermatophore extrusion in copulation, after subgenital plate opening, after spermatophore protrusion, and an arbitrary time after the start of courtship before copulation. Dissection was made soon after freezing the males in liquid nitrogen (−192°C).

Ablation of sensory organs and reproductive organs

To block visual input, the compound eyes and oscelli were covered by black lacquer. To reduce chemical input, the antennae, palpi and tarsi were cut off with scissors. For reproductive organs, the accessory glands or the testes were removed with a pair of forceps after opening the cuticles of the respective abdominal segments. This was done on day 3 after molt for the males to recover sufficiently before testing.

Transection of the nervous system

After the male copulated, it was anesthesized by cooling at 4°C, fixed on a cork board, and the cuticles over the intended level of the connectives or brain were cut (Fig. 9 inset). The connectives were cut at different levels; between the brain and the suboesophageal ganglion (Fig. 9e), between the metathoracic ganglion and the 1st abdominal ganglion (Fig. 9c), and between the 4th abdominal ganglion and the 5th abdominal ganglion (Fig. 9b). The connectives between the suboesophageal ganglion and the prothoracic ganglion was separated by cutting off the head at the neck (decapitation Fig. 9d). The brain was cut 3 ways with a razor blade; cut horizontally to separate the anterior region (Fig. 9f), cut vertically at the midline to separate it into the left and right (Fig. 9j), or cut diagonally to separate the antero-lateral regions (Fig. 9h) or the antero-median region including the pars intercerebralis (Fig. 9g). The corpora allata and the corpus cardiacum were removed together with forceps (Fig. 9i). Control males were only anesthetized for the same duration as transected males. All tests started on the day of surgery.

Spermatophore checking in nervous system transected males

To examine the operated male's ability to produce the spermatophore, the interior of the subgenital plate was checked every 6 hr for 3 successive days during which each male was kept isolated in a 200 ml beaker. A spermatophore was marked by India ink to discriminate from a new one. The number of males that produced spermatophore(s) during the 3 days, and the number of spermatophore(s) produced per male per day were counted.

RESULTS

Process to spermatophore protrusion

Behavior

Main reproductive events are shown in abbreviations in the middle of Fig. 2. The male copulates with the female some time after pairing (MFP). When the genitalia separated after spermatophore extrusion (GS in Fig. 2), all bodily movements stopped. In 1 sec (C.I. 1–2, n=21), a half (51%, n=41) of the males, then, exhibited post-copulatory body thrust [5] similar to copulatory movement for 18 sec (C.I. 14–30, n=21). These movements occasionally continued for up to a few minutes interposed by short rests. It finally ceased when the male lowered its abdomen itself (71%, n=21) but, in the remaining 29%, the body thrust was terminated by the female's movements on the male's back. Then, the male crept forward from under the female with his body rocking in a typically aggressive expression (MFS in Fig. 2). A distribution of the time interval between genitalia separation (GS) and male-female separation (MFS) is shown in Fig. 2A. The GS-MSF interval distributed from 5 to 80 sec with a median value of 18 sec (C.I. 8–23, n=29). The interval (30 sec (C.I. 6–43, n=21)) of males exhibiting body thrust was significantly longer than that (8 sec (C.I. 4–12, n=20)) of those not exhibiting body thrust. This post-copulatory body thrust developmentally emerged on day 4th after molt [5] and may play a role in keeping the female quiet longer to secure the settlement of the spermatophore in the genital chamber of the female.

After male-female separation, the male exhibited watching behavior [12] or guarding behavior [3]. Occasionally two kinds of characteristic movements were observed; one, abdominal contraction similar to those seen in evacuation of feces in a strained posture and the other, rapid antennal vibration at 10 Hz in the antero-postero direction [13]. One example of the occurrences of these movements in the male is shown by bars on the two lines in the middle of the Figure 2. The frequency of the abdominal contraction reached a maximum around spermatophore protrusion. It may reflect the activity of the reproductive organs to prepare a new spermatophore or warm-up for its ejection. Shortly before the spermatophore was ejected, the male often repeated 2–3 small openings of the subgenital plate. Then, the plate was widely pulled down (Fig. 2B inset) referred to here as subgenital plate opening (SGPO). The interval between male-female separation (MFS) and subgenital plate opening (SGPO) was 4 min (C.I. 3–9, n=29, Fig. 2B).

Then, the abdominal contraction with the backward pushing of the genitalia complex became frequent and the immature spermatophore was ejected through the genital opening to the space between the ventral lobes, that is, the moment of spermatophore protrusion (Fig. 2C inset). The interval between subgenital plate opening (SGPO) and spermatophore protrusion (SPPT) was 45 sec (C.I. 43–49, n=28), very constant for its shortness with a small C.I. (Fig. 2C).

Timing of spermatophore materials secretion into the ejaculatory duct

Prior to spermatophore protrusion, the mate-

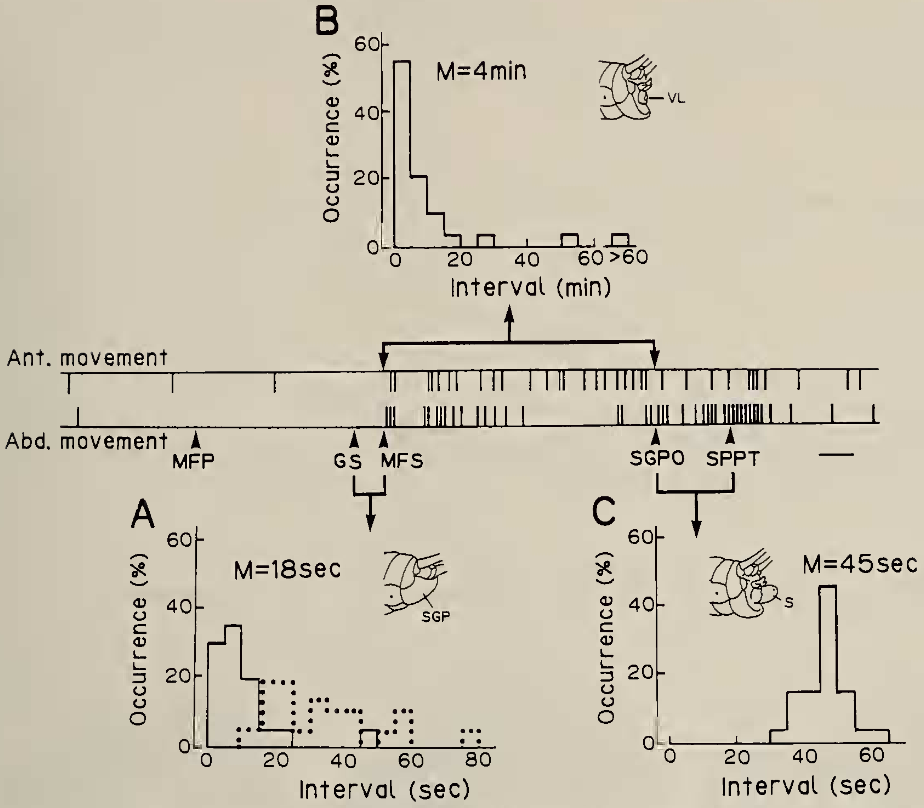

FIG. 2. Reproductive events and time intervals between them. In the center, the occurrences of antennal (upper) and abdominal (lower) movements shown by vertical bars. The male was paired with a female at MFP. Scale bar, 10 sec. A-C: histograms of the time intervals. Time bin, 5 sec in A and C, and 5 min in B. A: distribution of the time interval between genitalia separation (GS) and male-female separation (MFS). Solid line, males (n=20) not exhibiting post-copulatory body thrust. Dotted line, males (n=21) exhibiting post-copulatory body thrust. B: distribution of the time interval (n=29) between MFS and subgenital plate opening (SGPO). C: distribution of the time interval (n=28) between SGPO and spermatophore protrusion (SPPT). The histogram representing >60 min in B indicates males which failed to exhibit SGPO within the time limit. Some of these conventions are adopted in Fig. 4. M, median value; S, spermatophore; SGP, subgenital plate; VL, ventral lobes.

rials of the spermatophore are secreted from the testes and accessory glands into the ejaculatory duct. To determine this timing, the inside of the ejaculatory duct was examined using males quickly frozen at different phases in the male cycle. The spermatophore was not found in any phase except the very short period between the subgenital plate opening and spermatophore protrusion (Table 1). This means that spermatophore formation is initiated at the moment of subgenital plate opening. Some critical observations are shown in Fig. 3. The ejeculatory duct was vacant in males in the period between the end of copulation and subgenital plate opening (Fig. 3A). Ten seconds after subgenital plate opening, a white mass containing spermatozoa and the pressure body are seen (Fig. 3B). Thirty seconds after subgenital plate opening, the spermatophore moves toward the genital opening (Fig. 3C).

We observed that a white mass emerged from the accessory glands into the vacant ejaculatory duct in the male in phases other than the one between subgenital plate opening and spermatophore protrusion when the dissected male was left to thaw at room temperature. This curious phenomenon seems to be an artifact caused by a

Table 1. Presence or absence of the spermatophore in the ejaculatory duct and ventral lobes

Time after reproductive events		Ejaculatory duct	Ventral lobes	n
Spermatophore extrusion	0	×	×	10
	10 (min)	×	×	5
	30	×	×	5
Subgenital plate opening	0	○, ×	×	10
	10 (sec)	○	×	10
	30	○	×	5
Spermatophore protrusion	0	×	○	10
	10 (min)	×	○	5
	30	×	○	5
Calling song	>0 (min)	×	○	10

× absence of spermatophore. ○ presence of spermatophore. ○, × presence of spermatophore in only 4 males. n, number of males used.

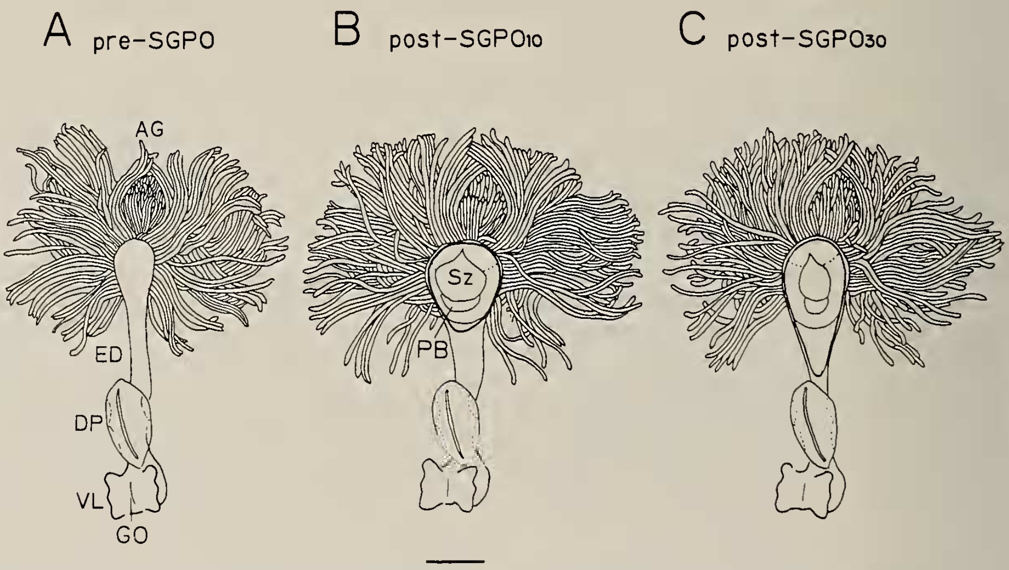

Fig. 3. Timing of the emergence of the spermatophore in the ejaculatory duct. Insects were frozen at 3 different phases in the male cycle. A: between the end of copulation and subgenital plate opening (SGPO). B: 10 sec after SGPO. C: 30 sec after SGPO. AG, accessory glands; DP, dorsal pouch; ED, ejaculatory duct; GO genital opening; PB, pressure body; Sz, spermatozoa; VL, ventral lobes. Scale bar, 1 mm.

loosening of the accessory glands due to tissue destruction.

Role of the female in spermatophore protrusion

Removal of the female during testing

To examine whether the female plays a major role in intiation of spermatophore protrusion, CPPT was measured in 3 different conditions (see methods); "male and female" (Fig. 4A), "male and male" (Fig. 4B) and "male" (Fig. 4C). In A as a control, 82% (n=32) of the males showed spermatophore protrusion within 10 min. The median of CPPT was 6 min (C.I. 5–8) with 3 min being the

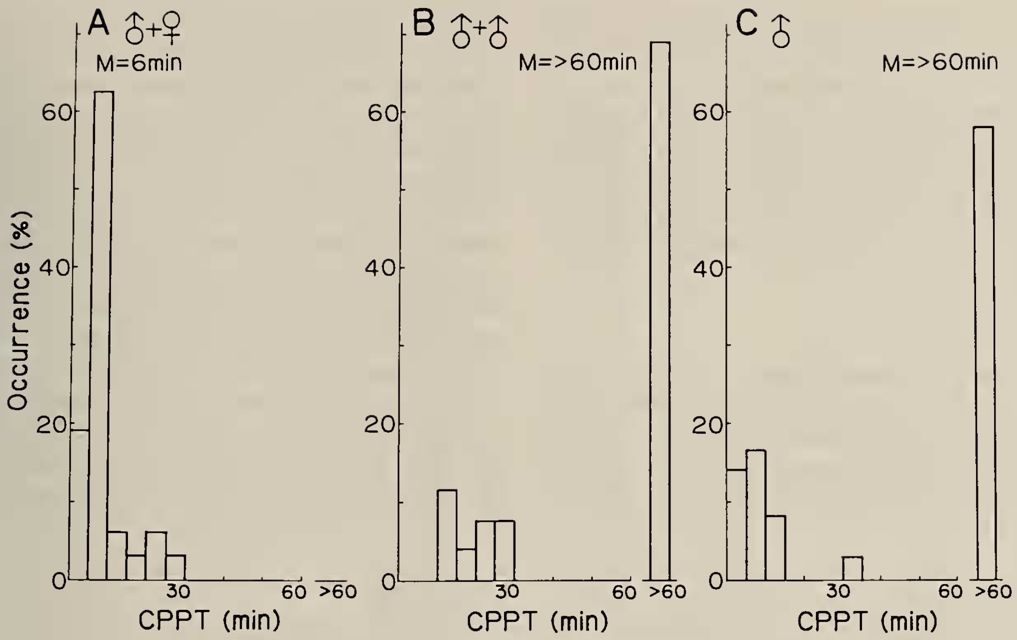

FIG. 4. Effect of the female presence after copulation on CPPT. A: control ("male and female", n=32). B: the male paired with a male after copulation ("male and male", n=26). C: the male separated from the female after copulation ("male", n=36). Time bin, 5 min.

shortest. In contrast, the male paired with a male after copulation made spermatophore protrusion in only 31% (n=26) within 1 hr (B). They were often fighting at the beginning. No males showed a CPPT shorter than 10 min. In C, 42% of males (n =36), which were kept quiet with no female after copulation, made spermatophore protrusion with a CPPT of >60 min (C.I. 12–>60). Twenty five percent of them showed a CPPT shorter than 10 min. There was a significant difference between B and C in the number of males exhibiting spermatophore protrusion within 1 hr. In B and C, males tend to split into two groups: those exhibiting spermatophore protrusion within half an hour and those not exhibiting it within 1 hr. In sum, these results reveal that spermatophore protrusion is considerably facilitated by the presence of the female.

Then, the effect of the female's presnece on CPPT was examined by changing the duration of pairing after copulation. Figure 5 shows the results. CPPT did not significantly decrease until the duration of the female presence was prolonged to 5

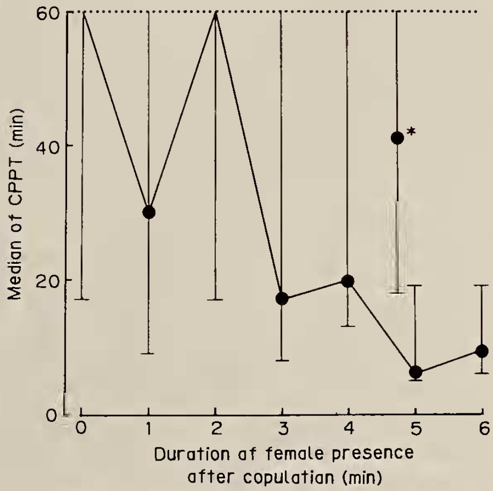

FIG. 5. Time dependence of female presence on CPPT. The abscissa represents the duration (min) of the presence of the female after copulation in the beaker. Asterisk, the male paired with a female again for only 1 min between 4 and 5 min after copulation. Each point represents the median for 30 males.

min (C.I. 5–19, n=30). However, this may be due to the change in male's internal state starting several min after copulation. To examine this possibility, the male was paired with a female for only 1 min between 4 and 5 min after copulation (asterisk). This short presence of the female was not sufficient to facilitate the initiation of spermatophore protrusion as seen in the CPPT of 41 min (C.I. 18–>60, n=30).

Block of sensory input

To study the nature of female stimulation, visual or chemical input was eliminated. CPPT in blind males (E in Fig. 6A) was 5.5 min (C.I. 3–7, n=20) which was not significantly different from that of intact males (Int in Fig. 6A). However, CPPT in males with antennae, palpi and tarsi removed (A, P & T in Fig. 6A) was 12 min (C.I. 7–23, n=25) which was much shorter than that of males tested without a female (Fig. 4C) but significantly longer than that of the intact males. This means the shortening of CPPT still occurred *via* male's chemoreceptors on the body surface as well as those on the antennae, palpi and tarsi. To determine whether receptors were olfactory or taste, the female was suspended by a string 8 cm over the male without contact (non-C in Fig. 6B). CPPT was >60 min (C.I. 18–>60, n=28). For control, a female was suspended 3 cm above the male allowing contact with her body. CPPT was 10.5 min (C.I. 5–18, n=20) which was much shorter than that of the non-C group and not significantly

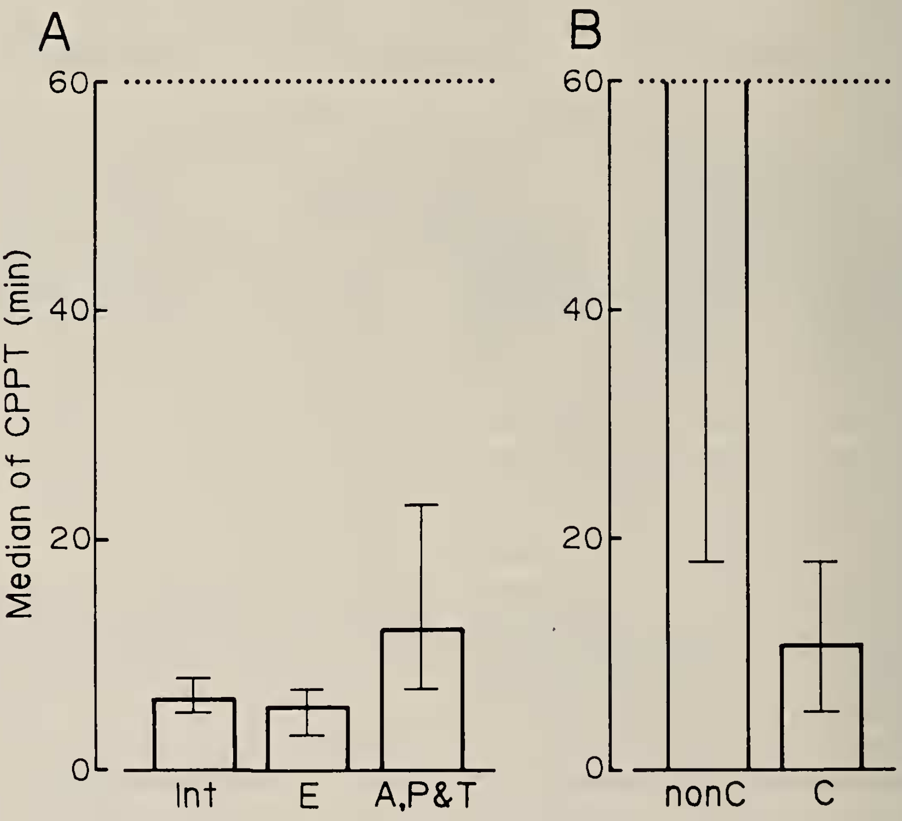

Fig. 6. Analysis of female factors. A: the effect of ablation of sensory organs. Int, intact (n=32). E, blind males (n=20) whose compound eyes and oscelli were covered with black lacquer. A, P & T, males (n=25) whose antenna, palpi and tarsi were removed. Note CPPT in A, P & T is much shorter than that of non-C, but still significantly longer than that of the Int. B: the effect of contact with the female. non-C, males (n=28) which could not contact with the female. C: males (n=20) which could contact with the female. A female was suspended 8 cm (non-C) and 3 cm (C) above the male, respectively.

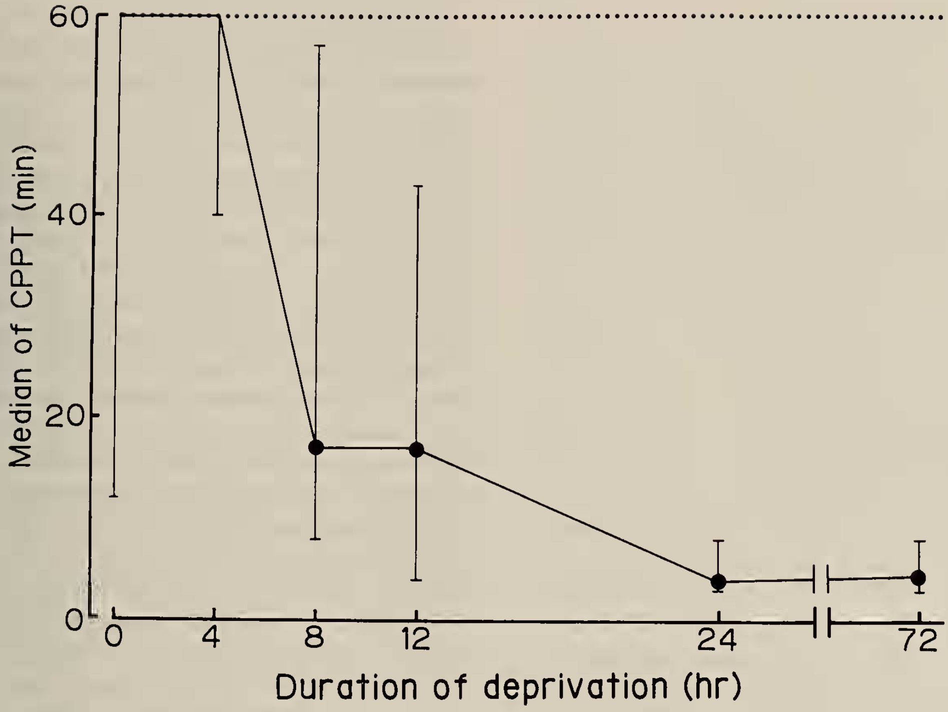

FIG. 7. Effect of female-deprivation prior to testing on CPPT. The abscissa represents the time (hr) during which males (n=30) had been separated from females prior to testing. Note the long latency at 0, 4, and 8 hr is characteristic of males tested in the "male" condition as in Fig. 4C.

longer than that of the intact group (Fig. 6A), This result reveals that female stimulation is not olfactory, but presumably contact-mediated.

Female-deprivation prior to testing

Males were temporarily separated from the females for a fixed time prior to pairing since CPPT was shorter in female-deprived males during the sexual maturation process after molt (Fig. 1B, C). During testing, CPPT was measured in the "male" condition so that the results would be much clearer than in the "male and female" condition because CPPT was expected to be shortened by deprivation (compare Fig. 4A with Fig. 4C). As shown in Fig. 7, the control (0 hr deprivation group) had a median value of >60 min (C.I. 12->60, n=36) similar to that of Fig. 4C. The CPPTs for the 4 hr and 8 hr deprivation groups were >60 min (C.I. 40->60, n=27) and 17 min (C.I. 8–57, n=31) respectively which were not significantly different from that of the control partly because of the large C.I.s. However, CPPT for 12 hr was 17 min (C.I. 4–43, n=27) which was significantly shorter than the control. At 24 hr, CPPT reduced markedly, as small as 4 min (C.I. 3–8, n=29). The interval, however, did not further decrease in the additional 48 hr of deprivation since CPPT was 4.5 min (C.I. 3–8, n=30) at 72 hr.

The shortening of CPPT may be due to a reduction of spermatophore production as a result of no copulation in the container. To test this possibility, males with accessory glands removed were used. The accessory gland ablated males could not make spermatophores but exhibit a spermatophore protrusion-like behavior [1] sometime after a vaccum copulation with the female [2–5]. Thus, CPPT was defined here as the time between genitalia separation and spermatophore protrusion-like behavior. Fig. 8 shows the results of the female-deprivation test in the "male" condition using accessory gland ablated males. CPPT of the control (AG (0)) was 47 min (C.I. 10->60, n

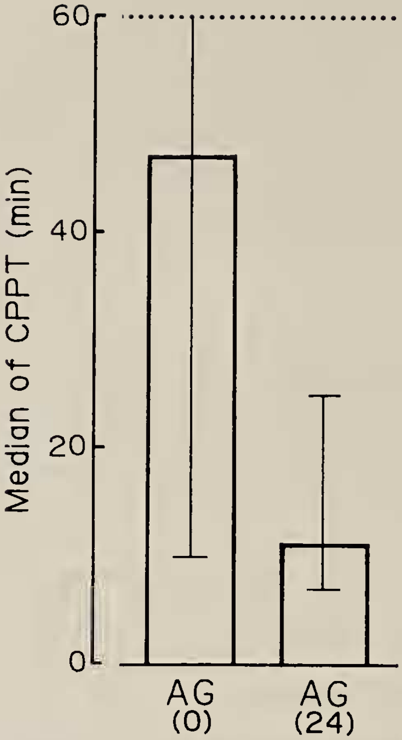

Fig. 8. Effect of female-deprivation in males with accessory glands removed on CPPT. AG(0), males (n=30) with no deprivation as control. AG(24), males (n=25) with 24 hr deprivation.

=30) while that of the 24 hr deprived group (AG (24)) was 11 min (C.I. 7–25, n=25). It is obvious that the shortening of CPPT occurred even in the males lacking the ability of spermatophore production. In addition, males whose testes were removed showed a similar result with a CPPT of 7 min (C.I. 5–8, n=36). These results indicate that the effect of female-deprivation can not be correlated with the lowered activity of spermatophore production.

The shortening of CPPT may be due to the lack of genitalia coupling [1] and subsequent spermatophore extrusion. Males were then placed with females for 24 hr whose subgenital plates were closed with wax to prevent males from hanging the epiphallus onto the female subgenital plate but not to prevent other mating acts. They were restricted in only genitalia coupling and spermatophore extrusion. The result indicated that the shortening of CPPT did not occur (CPPT>60 min (C.I. 28->60, n=20)). This revealed that the shortening of CPPT was not correlated with the inability of the genitalia to couple and to extrude the spermatophore.

It is worth mentioning that males reared with males (they were not used so far in the experiments) occasionally showed an extremely short CPPT when they were tested in the "male and female" condition. Although it was not apparent in the males in Fig. 1C, in one case using a different batch, CPPT was as short as 3 min (C.I. 3–4, n=28). The shortest of these was 1 min. Such a short CPPT was seldom observed in males reared with females (Fig. 1A). This reveals that the effects of the female-chemical substances and female-deprivation could be additive.

Effect of restraint in spermatophore protrusion

Table 2 shows the effects of the forced posturing on spermatophore protrusion. Sixty-five per cent of the "posture normal, leg contacted" males exhibited spermatophore protrusion with a relatively long latency (40 min (C.I. 19->60)), while only 10% of the "posture normal, leg restrained" males did so. In contrast, no males of the "posture reversed" groups ejected the spermatophores within a 1 hr and the following 24 hr. These results indicate that the male must be kept in a normal posture to protrude the spermatophore with the legs freely moving in contact with the substrate. In an additional experiment, the male was immediately paired with a female in a beaker at the end of stress testing ("posture reversed, legs suspended"). They showed spermatophore protru-

Table 2. Spermatophore protrusion under restrained conditions

Conditions		Spermatophore protrusion (%)	CPPT (min)
Posture	Legs		
Normal	Contacted	65 [30]	40 (C.I. 19->60)
	Restrained	10 [35]	18, 55
Reversed	Contacted	0 [0]	—
	Suspended	0 [0]	—

Number in each group, 20. Number in square brackets, males produced spermatophore(s) within 24 hr after the end of a 1 hr observation.

sion with a CPPT of 9 min after pairing (C.I. 8–17, n=20) which was not significantly different from the control (8 min (C.I. 8–10, n=33)) tested simply under the "male and female" condition using the same group. This indicates that spermatophore protrusion was inhibited during heavy stress but there was no aftereffect.

Transition from SGPO to SPPT

Disturbance

As shown in Fig. 2-C, the fact that the interval between subgenital plate opening and spermatophore protrusion was very constant suggested that both actions were linked together. Thus, whether spermatophore protrusion can be prevented by heavily distracting the male immediately after subgenital plate opening was examined. The male was quickly picked up at subgenital plate opening and kept gripped in the palm. In spite of such a harsh treatment, all the males showed spermatophore protrusion in the normal time interval of 45 sec (C.I. 42–51, n=25).

Transection of the connnectives

To estimate the involvement of the anterior nervous system in initiation and execution of spermatophore protrusion, the connectives were cut at the neck by decapitation or at the junction between the thorax and the abdomen immediately after subgenital plate opening. Both the groups showed spermatophore protrusion with the normal time interval of 45 sec (C.I. 41–48, n=20) and 45 sec (C.I. 42–51, n=20), respectively. These results revealed that spermatophore protrusion is an unstoppable process once the subgenital plate opens and is accomplished under the control of only the abdominal ganglia. In contrast, as will be described below, when the connective transection was made before subgenital plate opening, spermatophore protrusion within the normal time range was prevented.

Effects of nerve and brain transection

When the male was kept with a female in a beaker, spermatophore protrusion occurred regularly after every copulation. The number of spermatophores reached an average of 16 per day in males on day 10 after molt [5]. On the other hand, when the male was kept alone in the beaker, it showed spontaneous cycle renewal [1] in which the male exhibited spermatophore protrusion without any mating actions. In the present experiments, 96% of the control males (Fig. 9, a) produced one or two spermatphores per day with an average of 1.6±0.7. No males (Fig. 9, b) with the bilateral connectives cut between the 4th and the 5th abdominal ganglion produced spermatophores. Only males in this group showed feces discharge all the time indicating that they had problems in the control of intestinal and abdominal movements. In contrast, males with a unilateral connective cut at the same level produced spermatophores similarly to the control. In males (Fig. 9, c) with bilateral connectives cut between the thorax and abdomen, the average number of spermatophores was about half the control. Unilateral transection again altered neither the number of the males producing spermatophores nor the number of spermatophores produced.

Decapitated males (Fig. 9, d) and decerebrated males (Fig. 9, e) also showed a significant decrease in the numbers of males producing spermatophores (71% and 50%) and the number of spermatophores produced (1.0±1.4 and 0.4±0.5) compared with the control.

The brain was cut in different ways to separate the particular regions. Horizontal transection (Fig. 9, f) caused a significant decrease in the numbers of males producing spermatophores (65%) and spermatophores produced (0.6±0.8). Males with the antero-median region eliminated (Fig. 9, g) had a significant decrease in both the numbers of males (68%) and spermatophores (0.5 ±0.6). On the other hand, males with the antero-lateral regions eliminated (Fig. 9, h) showed only a significant derease in the number of spermatophores (0.8±0.6) with a similar number of males producing spermatophores (79%) as the control. The median transection (Fig. 9, j; n=22) caused a significant decrease in the number of spermatophores produced but not in the number of males producing them (88%). Finally, the removal of the corpora allata and the corps cardiacum (Fig. 9, i; n =12) did not alter the number of males (91%) or spermatophores (1.3±0.8).

Only the disconnection of the bilateral nerve

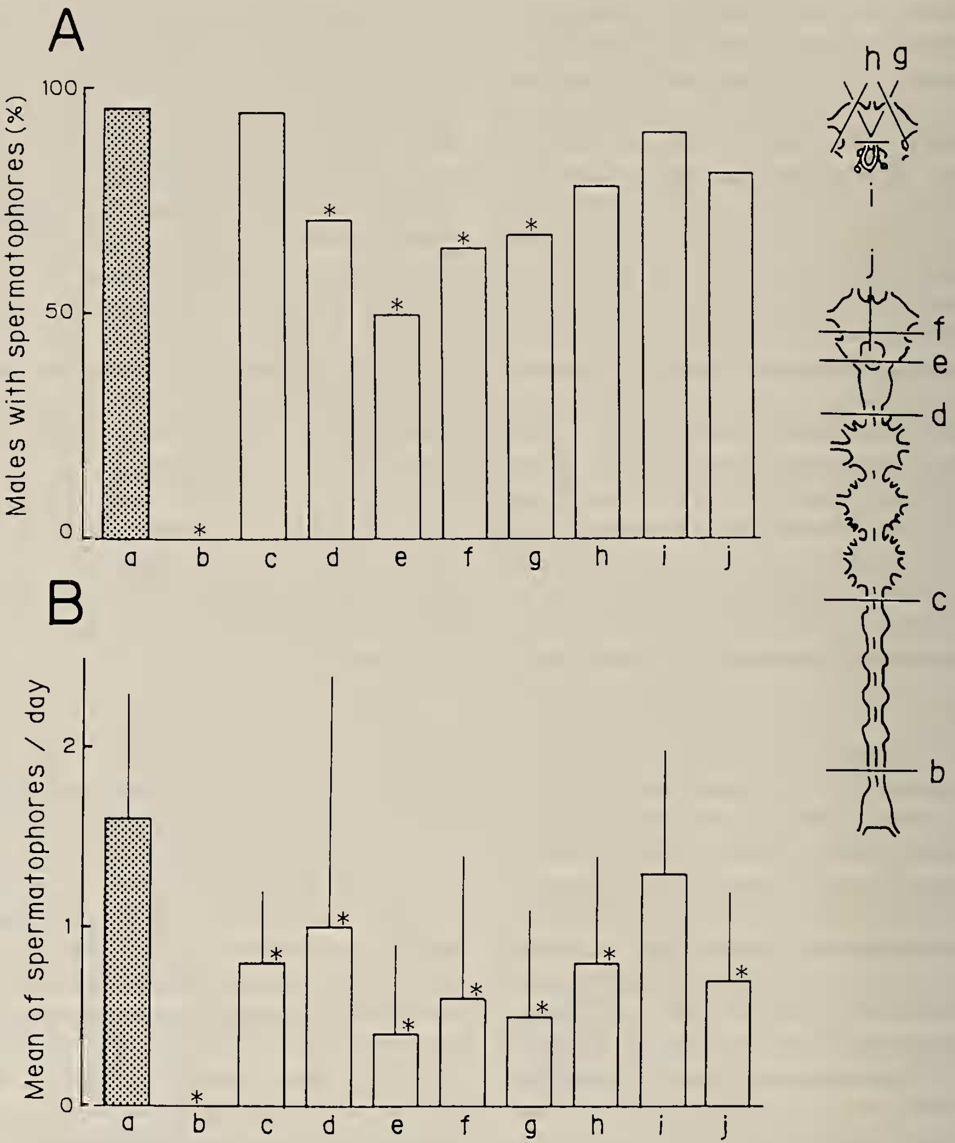

FIG. 9. Effects of transection of the connectives, separation of the brain, and removal of the corpora allata and corpus cardiacum. A: percentage of males producing mature spermatophores at least once in 3 successive days following surgery. B: the average number of mature spermatophores produced per male per day. Small letters (b-j) represent the types of surgery corresponding to those in inset. Each group consisted of the following number of males; 24(a), 13(b), 20(c), 14(d), 12(e), 20(f), 22(g), 19(h), 12(i), and 22(j). Asterisks indicate significant differences (A: χ^2 test, $P<0.05$; B: t-test, $P<0.05$) between (a=control) and (b-j).

cords between the 4th and the 5th abdominal ganglion abolished spermatophore protrusion. These results indicate that spermatophore protrusion can be initiated and executed without any influence from the head or throacic ganglia.

DISCUSSION

The present study shows the starting event of spermatophore protrusion, and external and internal factors that influence the latency of spermatophore protrusion in the male cricket *Gryllus* *bimaculatus.* Initiation of spermatophore protrusion is considerably facilitated both by contact with the female and by an increase in sensitivity to the female. On the other hand, spermatophore protrusion was delayed or prevented under stressful conditions such as persistent restraint in an abnormal posture. Spermatophore protrusion could occur, though more slowly, in males with the connectives cut, except connectives between the 4th and the 5th abdominal ganglion. Our results provide the basis for future neurophysiological study to elucidate the initiation mechanism of spermatophore protrusion.

Start of spermatophore production

Spermatophore protrusion occurs with a more variable CPPT compared with that (about 1 hr) of the calling song [1]. In extreme cases, CPPT was more than 1 hr (see Fig. 2B) while only 1 min at the shortest. These findings suggest that the process of spermatophore protrusion is influenced by some external and internal factors in contrast to the process of the calling song which is dependent on an internal timer [4].

Spermatophore protrusion occurs 45 sec after subgenital plate opening. Transection of the connectives revealed that these two actions were a continuous process. Inspection of the spermatophore in the ejaculatory duct indicated that the secretion of sperm from the testes and proteinous substances from the accessory glands occurred at the moment of subgenital plate opening. These facts indicate that spermatophore protrusion, which we defined *a priori* as the moment of immature spermatophore ejection through the genital cavity to the exterior, actually starts at the moment of accessory gland contraction to secrete the spermatophore materials into the ejaculatory duct [14] possibly mediated by dorsal unpaired median (DUM) neurons [15]: i.e. the male cycle starts at the time of subgenital plate opening. Previously, one paper [4] reported that a new spermatophore was already present in the ejaculatory duct soon after spermatophore protrusion and stayed there until the next spermatophore protrusion, while the other paper [14] did not confirm this and rather agreed with our present result. We suspect the observations by the former authors might be an artifact due to freezing and thawing.

Facilitation of spermatophore protrusion

CPPT was much shorter in the male kept continuously with the female after copulation. This suggests that some contact-mediated chemical substances are facilitating spermatophore protrusion. Chemo-receptors mediating spermatophore protrusion may be distributed over the body surface because the shortening of CPPT was still apparent in males without the antennae, palpi and tarsi. Previously in *Teleogryllus,* the female had no effect on spermatophore protrusion [3] though its contact chemoreceptors certainly facilitated mating actions [16]. The discrepancy may be due to the difference in species or the difference in the size of the space where animals were tested. The compartment they used was a little wider than our 200 ml beaker, which might have lowered chances of contact with each other. It is interesting that males split into those with CPPT less than 30 min and those with more than 1 hr when tested in no female conditions (Fig. 4B, C). Probably an individual difference in male reproductive activity was exaggerated through some accommodation which increased the threshold of spermatophore protrusion under less stimulative conditions.

Spermatophore protrusion was also facilitated after males were temporarily kept isolated from females. This was already seen from day 3 after molt, and matched our empirical observations that males reared without females were sexually more excitable than those with females [1]. Our systematic study of female-deprivation found that the shortening of CPPT became pronounced after a 12–24 hr separation. Our results indicated that this effect was neither due to a reduction of spermatophore production nor due to the lack of genital coupling and spermatophore extrusion during isolation. It is probably caused by an increase in sensitivity to the chemical substances of the female.

Inhibition of spermatophore protrusion

Stress, such as forced posturing, significantly delayed or even prevented spermatophore protrusion. In particular, males fixed in the upside-down position were unable to push out the spermatophores, indicating that normal posture with the legs kept freely on the substrate is a prerequisite. A similar result was previously obtained by fixing the body to a board with paraffin [4]. These results give cautions against neurophysiological study where animal restraint would be inevitable. Similar but not large delay in CPPT was also found under a different type of stress, i.e., fighting with a male (Fig. 4B).

It should be worth comparing the resistivity to stress between the two interval states: genitalia separation to spermatophore protrusion *vs.* spermatophore protrusion to calling song. As long as the male is under heavy stress, it is natural that the male cannot initiate either spermatophore protrusion or calling song. However, the male sang the calling song with the normal latency (about 1 hr) from spermatophore protrusion when stress was terminated in less than 1 hr after spermatophore protrusion [4]. Stress did not disturb the running timer. On the other hand, the male exhibited spermatophore protrusion with a latency of 1 h plus the normal CPPT when stress was terminated 1 hr after copulation and the male was paired with a female. Stress inhibited the processing necessary for spermatophore protrusion.

Role of the brain in spermatophore protrusion

It is known that nervous communication between the brain and the terminal abdominal ganglion is necessary for the male cricket to perform mating behavior normally [9]. Here, we state more concretely that male crickets with both connectives cut at the thoracic level can perform spermatophore protrusion but not produce as many spermatophores as intact males. This result means that the motor program for spermatophore production resides totally within the abdominal ganglia and can proceed without command of the anterior ganglia. Normally, however, spermatophore protrusion is certainly triggered by a command in the brain because decerebrated males failed in spermatophore protrusion within the normal time range after copulation.

It would be interesting to record the descending neural activity conveying signals for spermatophore protrusion which should arrive at the abdominal ganglia before subgenital plate opening. The fact that spermatophore protrusion required 45 sec from subgenital plate opening and that the minimum CPPT (genitalia separation to spermatophore protrusion) was only 1 min suggest that information on mating termination generated in association with the dorsal pouch contraction to extrude the spermatophore [1] can be processed immediately in the brain and in turn can trigger the pattern generator in the abdominal ganglia. At the same time, some neural events associated with DUM neuron activity for secretion of the spermatophore materials into the ejaculatory duct may set the timer for the calling song. The resultant accessory gland contraction itself or the ensuing spermatophore ejection to the external genitalia is not concerned with the timer setting since males without accessory glands [2] and those with spermatophores but incapable of spermatophore protrusion [1] still retain the normal timer function.

Regarding the effects of partial elimination of the brain, determination of brain regions critical for spermatophore protrusion was virtually impossible because the activity of spermatophore production in intact males was very low (1 or 2 per day) and that in the experimentals was not zero but 0–1 per day: the differences were too small to compare. The total inability of males with the connectives transected between the 4th and the 5th abdominal ganglion to perform spermatophore protrusion is probably due to the paralysis of abdominal segments as suggested by continuous discharge of feces. The results of the corpora allata and corpus cardiacum removal was in agreement with the previous report that elimination of either organ failed to abolish mating behavior [2].

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